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14. ABSTRACT

Metastatic castration-resistant prostate cancer (CRPC) is a major cause of prostate cancer-associated mortality. Genes/proteins that drive or sustain CRPC tumor growth and metastasis are promising targets for therapeutic intervention. EZH2 (enhancer of zest 2), an enzyme that catalyzes histone 3 lysine 27 trimethylation (H3K27me3), was found among the most up-regulated genes in CRPC and strongly promotes disease progression. Surprisingly, enzymatic EZH2 inhibitors such as EPZ-6438 (also called Tazemetostat, Epizyme) has limited efficacy in prostate cancer, suggesting that EZH2 may play non-catalytic roles in prostate cancer. Our preliminary data suggest androgen receptor (AR) as a critical target and mediator of the non-catalytic roles of EZH2. We found that EZH2 can directly induce the transcription of the AR gene and that enzymatic EZH2 inhibitors failed to block AR induction. We hypothesize that EZH2 plays dual roles in prostate cancer and that combination of EPZ-6438 with AR pathway inhibitors will be highly effective in fully blocking EZH2 function and thus suppressing CRPC.

15. SUBJECT TERMS
EZH2, EED, Prostate Cancer, AlphaScreen, fluorescence polarization, medicinal chemistry, PROTACS, covalent inhibitors

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TABLE OF CONTENTS

	Page
1. Introduction	**4**
2. Keywords	**4**
3. Accomplishments	**4-12**
4. Impact	**13-14**
5. Changes/Problems	**15**
6. Products	**16-17**
7. Participants & Other Collaborating Organizations	**18-20**
8. Special Reporting Requirements	**20**
9. Appendices	**20-21**

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

It has been well-established that the methyltransferase EZH2 plays a critical role in castrate-resistant prostate cancer (CRPC). We and others have shown that EZH2 possesses previously uncharacterized roles that are independent of the PRC2 complex and/or independent of its methyltransferase activity. While there have been a number of catalytic EZH2 inhibitors developed, none of these is capable of blocking these non-catalytic oncogenic functions. Our overall goal is to develop small molecules that inhibit both the catalytic and non-catalytic functions of EZH2, and thereby develop a new treat CRPC by blocking its oncogenic functions. Our overall approach is to integrate in vitro screening, medicinal chemistry, and molecular modeling to identify and develop compounds that inhibit the binding between EZH2 and EED, which will lead to EZH2 degradation and prevent its oncogenic functions.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

EZH2, EED, Prostate Cancer, AlphaScreen, fluorescence polarization, medicinal chemistry, PROTACS, covalent inhibitors

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Investigate a non-catalytic role of EZH2 in directly inducing AR gene transcription.	**Timeline**	**Site 1** (Initiating PI)	**Site 2** (Partnering PI)	**% of completion**	**Actual complete dates**
Major Task 1: Demonstrate that EZH2 directly activates AR gene transcription.	Months				
Subtask 1: To demonstrate that EZH2 protein occupies the AR gene promoter.	1-6	Dr. Yu		100%	12/01/2017
Subtask 2: To pinpoint the EZH2 binding elements at the AR gene promoter using ChIP-qPCR in combination with CRISPR-Cas9-mediated deletion.	1-12	Dr. Yu		60%	
Subtask 3: To show that EZH2 binding is required for AR promoter activity and AR gene expression using luciferase assay and CRISPR-Cas9-mediated deletion, respectively. Cell lines used 293T, LNCaP, C4-2B, and 22Rv1 (ATCC)	3-18	Dr. Yu		100%	01/31/2018

Major Task 2: Determine whether EZH2 activates AR independently of PRC2 and its methyltransferase activity.					
Subtask 1: Perform ChIP and re-ChIP of EZH2, SUZ12, EED, and H3K27me3 at the AR promoter.	1-12	Dr. Yu		100%	07/31/2018
Subtask 2: Examine how enzymatically inactive EZH2-H689A mutant regulates AR expression.	9-18	Dr. Yu		100%	07/31/2018
Subtask 3: Determine whether enzymatic EZH2 inhibitors fail to decrease AR expression. Cell lines used LNCaP, C4-2B, and 22Rv1 (ATCC)	9-18	Dr. Yu		100%	01/30/2018
Major Task 3: Identify potential EZH2 cofactors that activate AR gene transcription.					
Subtask 1: Determine which domains of EZH2 is required for AR activation.	9-24	Dr. Yu		20%	
Subtask 2: Comparative mass spec analysis to identify EZH2-interacting cofactors that activate AR.	12-24	Dr. Yu		20%	
Subtask 3: AR promoter motif analysis to identify potential upstream regulators of AR.	18-24	Dr. Yu		80%	
Milestone #1: Manuscript reporting RPC2-indepndent, non-catalytic roles of EZH2 in activating AR transcription.	1-18	Dr. Yu		80%	
Specific Aim 2: Examine dual roles of EZH2 in PCa and their therapeutic targeting in CRPC.					
Major Task 4: Delineate the non-catalytic roles of EZH2 in regulating downstream genes and pathways in PCa cells.					
Subtask 1: Determine genes differentially regulated by EZH2 wildtype and its enzymatically inactive EZH2-H689A mutant.	6-18	Dr. Yu		20%	
Subtask 2: Identify genes/pathways that are regulated by EZH2 knockdown but not by enzymatic EZH2 inhibitor EPZ-6438.	12-30	Dr. Yu		100%	07/31/2018
Subtask 3: Examine EZH2-regulated gene signatures in control and AR-depleted cells. Cell lines used LNCaP, C4-2B, 22Rv1, and NE1.3 (ATCC)	12-36	Dr. Yu		30%	
Milestone #2: Manuscript on dual roles of EZH2 in prostate cancer and their therapeutic targeting.	12-36	Dr. Yu		80%	
Major Task 5: Evaluate the efficacy of EPZ-6438 alone or in combination with Enzalutamide in preclinical models of PCa.					
Subtask 1: Examine combinatorial effects of EPZ-6438 and Enzalutamide in diverse PCa cell lines *in vitro*. Cell lines used LNCaP, C4-2B, 22Rv1, and NE1.3	6-30	Dr. Yu		70%	11/01/2017

(ATCC)					
Subtask 2: Evaluate the efficacy of EPZ-6438 alone or in combination with Enzalutamide in suppressing xenograft tumor growth *in vivo*. Cell lines used LNCaP, C4-2B, and 22Rv1 (ATCC)	9-36	Dr. Yu		70%	
Milestone #3: Submit a LOI for a phase I clinical trial of Enzalutamide and EPZ-6438 in CRPC patients.	12-36	Dr. Yu		10%	
Specific Aim 3: To develop next-generation small molecule EZH2 inhibitors capable of EZH2 protein degradation.					
Major Task 6: Complete virtual high-throughput screen to identify novel EZH2/EED inhibitors					
Subtask 1: Prepare ligand database and EED structure for virtual screening	1-3		Dr. Schiltz	100%	10/31/17
Subtask 2: Perform docking-based vHTS of the curated Zinc database against EED	3-5		Dr. Schiltz	100%	3/31/18
Subtask 3: Prioritize compounds and screen in vitro	5-9	Dr. Yu	Dr. Schiltz	20%	
Milestone #4: Identify experimentally validated novel EZH2/EED inhibitors	9		Dr. Schiltz	90%	
Major Task 7: Optimize the compound series into potent EZH2/EED inhibitors with reduced hERG liability					
Subtask 1: Design new astemizole analogs and derivatives of our vHTS hits with the aid of molecular modeling in the EED structure to help prioritize compounds for synthesis.	1-36		Dr. Schiltz	5%	
Subtask 2: Synthesize and purify approximately 10 new compounds per month.	1-36		Dr. Schiltz	10%	
Subtask 3: Screen new molecules in our screening funnel, including fluorescence polarization (FP), western blotting of EZH2, and then cell viability. Cell lines used DB and Toledo (ATCC)	1-36	Dr. Yu	Dr. Schiltz	20%	
Milestone #5: Develop analog(s) that are at least as potent as astemizole with hERG binding reduced by >100 fold. Develop novel inhibitors that have FP potency < 1uM.	24-36		Dr. Schiltz	10%	
Major Task 8: Evaluate the DMPK and binding properties of our most promising inhibitors					
Subtask 1: Evaluate the binding of potent inhibitors using NMR-based EED/Ligand binding studies.	1-36		Dr. Schiltz	0%	
Subtask 2: Screen potent inhibitors for hERG binding and in vitro DMPK properties.	1-36		Dr. Schiltz	0%	
Subtask 3: Evaluate the exploratory in vivo PK properties of promising inhibitors using RACE PK	12-36		Dr. Schiltz	0%	

studies.					
Subtask 4: Examine the efficacy of promising inhibitors in reducing EZH2 and suppressing prostate cancer cell growth *in vitro* and tumor growth *in vivo*.	24-36	Dr. Yu		20%	
Milestone #6: Manuscript and patent on 1-3 lead compounds that have < 1uM potency at EZH2/EED inhibition and cell viability, with satisfactory DMPK properties, tumor-inhibiting effects.	24-36	Dr. Yu	Dr. Schiltz	10%	

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. Major Activities

Our major activity during the first year was to use in vitro and in silico screening to identify compounds that disrupt the EED-EZH2 interaction to facilitate degradation of EZH2 and subsequent reduction of AR.

2. Specific Objectives

Our specific objectives were to identify and validate small molecule inhibitors that bound to EZH2 or EED and prevented their binding, thereby disrupting PRC complex formation. While a number of EZH2 catalytic inhibitors are known, none of these blocks the non-catalytic functions of this oncoprotein. Our overall objective is to develop new therapeutic strategies for treating EZH2-mediated prostate cancer by inducing its degradation, and thereby inhibit all of its pro-proliferative functions.

3. Significant results or key outcomes

Assay Development. We first developed two biochemical assays to help characterize ligand binding at the EED-EZH2 interface: 1) An alphaScreen assay using full-length EED and EZH2 and 2) fluorescence polarization (FP) assay using full-length EED and FITC-labeled EZH2 peptide.[1] Both assays were validated with known inhibitor, SAH-EZH2.[2] Optimal conditions for the AlphaScreen were identified by titrating FLAG-EED (aa 2-end) and His-GST-EZH2 (aa 2-end) in 1x HMT Assay Buffer 2 (BPS Bioscience) (Table 1). Similarly, the beads were titrated to reduce the cost of the assay. The optimized protocol required 1 h incubation of compound with 5 nM FLAG-EED and 8.3 nM His-GST-EZH2 followed by 1 h incubation with 0.1 µg and 0.05 µg of anti-FLAG AlphaScreen acceptor and glutathione donor beads, respectively. The signals were then read on an Enspire microplate reader.

Table 1. Alpha counts (n = 2)

FLAG-EED nM	GST-His-EZH2 26	8.7	2.9	0.96
5.0	323,030	**223,564**	52,233	11,602
1.7	121,155	176,880	62,539	5,747
0.56	79,795	74,537	52,346	2,944
0.19	31,414	12,852	4,859	3,218

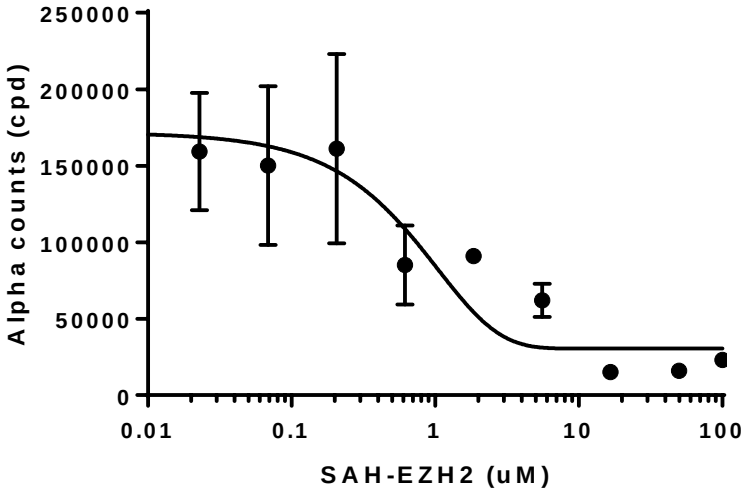

Figure 1. Titration with SAH-EZH2 (IC_{50} = 1.5 ± 0.67 µM).

To develop the FP assay, the N-terminal FITC-labeled EZH2 peptide (aa 40-63) was synthesized by the Peptide Synthesis Core at Northwestern University. The tracer was then titrated with FLAG-EED in 20 mM HEPES buffer at pH 8.0 with additives (150 mM NaCl, 0.1 mg/mL BSA, 0.01% NP40, and 4% DMSO) (Figure 2A). The final protocol required 2 h incubation of compound with 100 nM FITC peptide and 1.0 µM FLAG-EED. The signals were then read on an M1000 microplate reader (**Figure 2**).

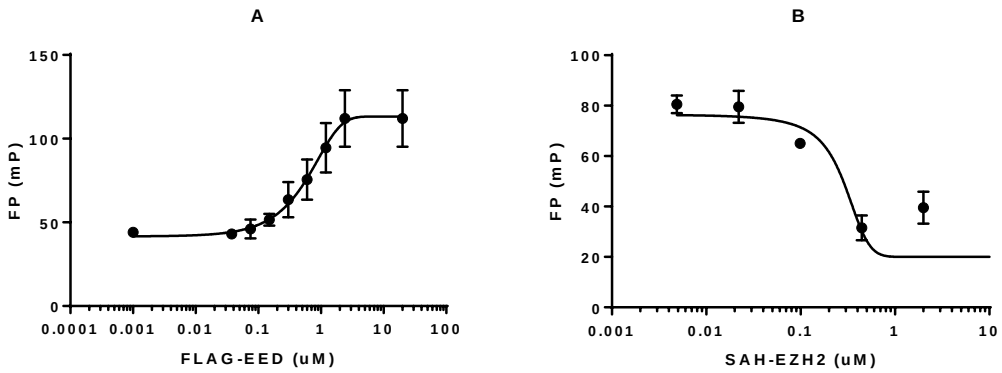

Figure 2. (A) Titration with FLAG-EED and 100 nM FITC-EZH2; (B) FP assay with SAH-EZH2 (IC_{50} = 2.1 ± 0.71 µM).

High-throughput Screening. We analyzed 10,000 compounds from the commercial Chembridge Diversity Library in the AlphaScreen assay using pools of 4. Deconvolution and dose-response testing led to identification of 46 potential hits (Table 2) with IC_{50} <170 µM. However, 39 of those compounds were identified as false positives in an AlphaScreen TruHits assay.

Table 2. First High-throughput Screening Results

	Alpha Screen IC_{50} (uM)	TruHits Pass or Fail?		Alpha Screen IC_{50} (uM)	TruHits Pass or Fail?
NUCCC-0159262	25.0	Fail	NUCCC-0171239	23.0	Pass
NUCCC-0149593	170.0	Fail	NUCCC-0140271	>170	Fail
NUCCC-0133313	150.0	Fail	NUCCC-0129046	40.0	Pass
NUCCC-0152125	5.1	Fail	NUCCC-0127684	12.0	Fail
NUCCC-0132344	>170	Fail	NUCCC-0132430	130.0	Fail
NUCCC-0162274	2.9	Fail	NUCCC-0150041	93.0	Fail
NUCCC-0137346	85.0	Fail	NUCCC-0160134	78.0	Pass
NUCCC-0154148	15.0	Fail	NUCCC-0142174	68.0	Pass
NUCCC-0138110	120.0	Fail	NUCCC-0151115	15.0	Fail
NUCCC-0164477	110.0	Fail	NUCCC-0159555	6.1	Fail
NUCCC-0165960	3.5	Fail	NUCCC-0126668	76.0	Pass
NUCCC-0163886	>170	Fail	NUCCC-0130433	11.0	Fail
NUCCC-0160587	15.0	Fail	NUCCC-0141985	38.0	Fail
NUCCC-0146892	10.0	Fail	NUCCC-0146784	18.0	Fail
NUCCC-0168310	9.2	Fail	NUCCC-0145198	>170	Fail
NUCCC-0168150	57.0	Fail	NUCCC-0146029	18.0	Fail
NUCCC-0155724	8.8	Fail	NUCCC-0174151	14.0	Fail
NUCCC-	120.0	Fail	NUCCC-	40.0	Fail

0130730			0163757		
NUCCC-0146331	40.0	Fail	NUCCC-0131489	12.0	Fail
NUCCC-0142808	>170	Fail	NUCCC-0133607	>170	Fail
NUCCC-0134009	43.0	Pass	NUCCC-0174163	25.0	Fail
NUCCC-0139869	57.0	Fail	NUCCC-0147446	17.0	Fail
NUCCC-0161072	39.0	Pass	NUCCC-0171968	21.0	Fail

We next analyzed the most diverse set of 10,240 compounds from the remaining library (~ 40,000 compounds) using the same AlphaScreen assay protocol to identify 16 additional compounds with low µM binding affinity (Table 3). Further analysis with the fluorescence polarization assay suggests that these compounds are binding at an unknown site that facilitates disruption of the EED-EZH2 complex.

Table 3. Second High-throughput Screening Results

	Alpha Screen IC_{50} (uM)	TruHits Pass or Fail?		Alpha Screen IC_{50} (uM)	TruHits Pass or Fail?
NUCC-0148600	15.3	Fail	NUCC-0130547	15.3	Fail
NUCC-0140518	9.1	Pass	NUCC-0132372	14.4	Fail
NUCC-0173093	17.2	Fail	NUCC-0144836	17.4	Fail
NUCC-0168988	16.1	Fail	NUCC-0157380	107.4	Pass
NUCC-0166393	2.5	Pass	NUCC-0147290	8.3	Fail
NUCC-0156742	0.2	Pass	NUCC-0174811	16.3	Fail
NUCC-0143506	47.9	Pass	NUCC-0133191	23.1	Fail
NUCC-0136764	33.0	Pass	NUCC-0145948	12.7	Fail
NUCC-0125673	18.3	Fail	NUCC-0150337	37.7	Pass
NUCC-0155629	0.6	Fail	NUCC-0147237	15.7	Fail
NUCC-0171144	38.0	Pass	NUCC-0161282	3.6	Pass
NUCC-0130760	0.4	Fail	NUCC-0145562	160.0	Pass
NUCC-0170505	16.5	Fail	NUCC-0174986	3.5	Fail
NUCC-0170748	12.5	Fail	NUCC-0148912	52.2	Pass
NUCC-0143771	5.1	Pass	NUCC-0154019	6.6	Pass
NUCC-0137018	38.8	Fail	NUCC-0168124	6.2	Pass
NUCC-0169860	24.4	Pass	NUCC-0168159	10.3	Fail
NUCC-0163358	6.4	Pass	NUCC-0131238	33.8	Fail
NUCC-0135753	6.9	Pass	NUCC-0154468	67.7	Pass
NUCC-0134680	21.2	Fail	NUCC-0173068	9.5	Fail

The leading 25 compounds from both HTS runs will be further evaluated and optimized via AlphaScreen and thermal shift assays before testing for cellular efficacy. Once we further validate our HTS hits, we will begin carrying out medicinal chemistry to improve their potency and pharmaceutical properties. We also plan to begin SAR on astemizole to improve the potency and solubility of the known ligand.[3]

To pursue alternative strategies to degrade EZH2, in addition to our HTS-identified EED-EZH2 inhibitors, we sought to develop new EZH2 degraders via 2 platforms: 1) cysteine-targeted covalent inhibitors and 2) Proteolytic Targeting Chimeras (PROTACs).[4-7] In 2017, GNA002 was reported to promote ubiquitin-mediated degradation of EZH2 at 2 µM by binding to Cys663 in the SET domain.[8] We synthesized a small series of 6 covalent inhibitors by incorporating an electrophilic moiety in known EZH2 catalytic inhibitor EPZ-6438.[9] The most potent compound, NUCC-0202059, degrades EZH2 and reduces AR at 2 µM in LNCaP cells after 48 h incubation (**Figure 3**). By contrast, its noncovalent counterpart, NUCC-0202139 only inhibits EZH2 enzymatic function at 2 µM. Furthermore, NUCC-0202059 inhibits cell proliferation in C4-2B cells, which are resistant to EPZ-6438 (**Figure 4**).

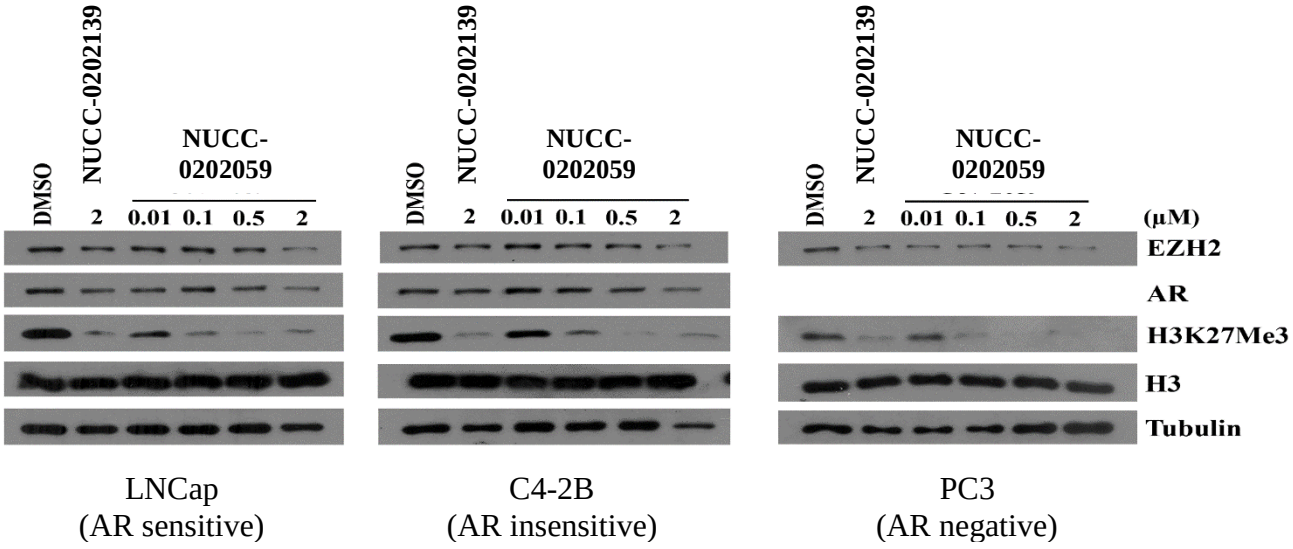

Figure 3. Western blots after 48 h incubation with NUCC-0202139 and NUCC-0202059.

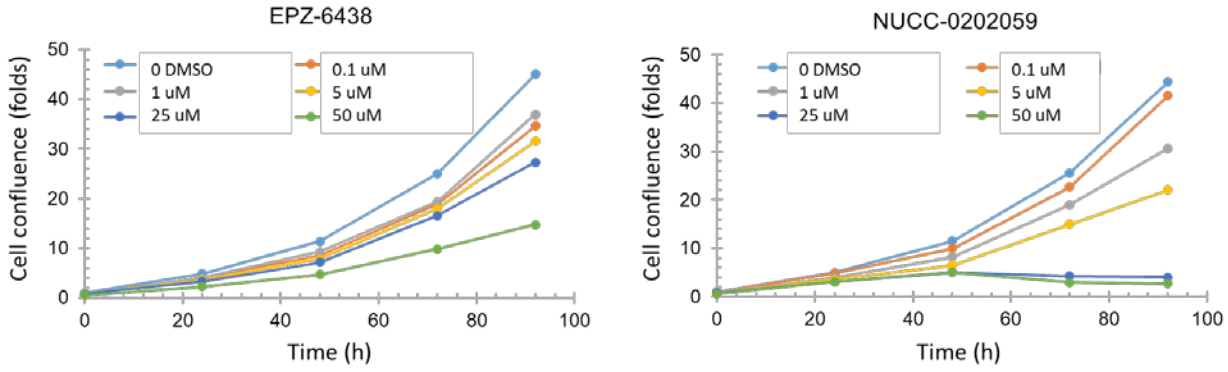

Figure 4. Cell proliferation with C4-2B cell line.

Finally, we have capitalized on PROTACS technology developed by Craig Crews to recruit an E3 ligase to mark EZH2 for proteolytic degradation. To date, we have developed 24 PROTACs based on known E3 ligands VHL and CRBN. Preliminary results illustrate that incubation with NUCC-0202099 and NUCC-0202100 degrade EZH2 and reduce AR (**Figure 5**). We are synthesizing the next generation of EPZ-6438 based PROTACs to improve potency.

In Silico-Screening. Using the published PRC complex crystal structure,[10] we carried out two in silico screens. Our focus was to identify compounds that bound EED where it interacts with the EZH2 alpha-helix, and thereby prevent their binding. Using a set of 10 Million structures previously filtered to possess good drug-like properties,[11-13] EED was subjected to a 3-tier docking protocol (Schrödinger Glide) for screening. The Glide high throughput virtual screening (HTVS) followed by Standard Precision (SP) and Extra Precision (XP) docking generated a virtual hit set of compounds. After manually visualizing the poses of the 100 highest-scoring ligands (each with a Glide XP score < -7.0), the initial hitset was filtered for druggability, synthetic tractability, and compound availability in a manner similar to recent work. This resulted in a set of about 50 compounds that were purchased for *in vitro* screening in our AlphaScreen and FP assays. This first in silico screen did not identify and inhibitors. We next carried out another in silico screen using a similar approach as above. However, in this screen, we focused on a slightly different ligand-binding position on EED. This resulted in another about 50 compounds that were purchased and screened in our assays. However, this also resulted in no inhibitors.

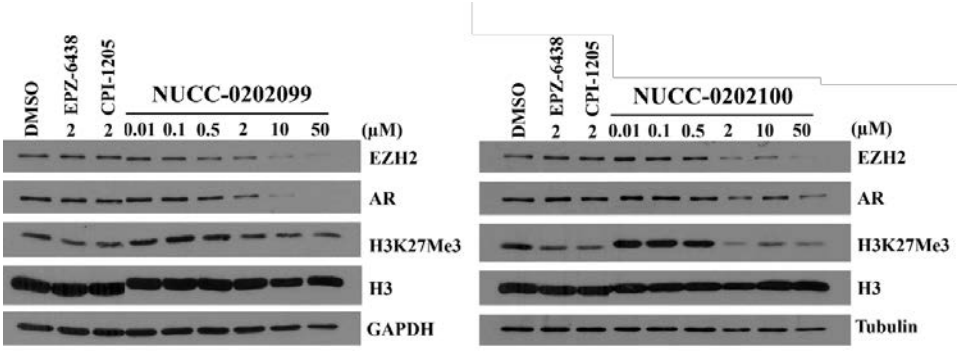

Figure 5. Western blots after 48 h incubation with NUCC-0202099 and NUCC-0202100.

4. Other Achievements

The above significant achievements section provides a review of all of our activities for the first year.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for

example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. Corinne Ley, a postdoctoral fellow, worked closely with scientists in the Northwestern University High-throughput analysis lab. Dr. Ley was trained on the use of several biological assay techniques and several analysis instruments (e.g. plate readers). She has attained strong proficiency in these techniques and has used her skills to carry out the in vitro alphascreen and fluorescence polarization assays.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Progress was presented by Dr. Ley with a poster at a Northwestern symposium titled "The Chemical Impact on Life Sciences Colloquium & Scientific Poster Session" Dr. Ley described our work so far using the PROTACS and covalent inhibitor strategies. Notably, Dr. Ley was awarded first place in the postdoctoral fellow category for her poster presentation.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

In the next year, we will continue our PROTACS and covalent inhibitor strategies by synthesizing and testing new analogs for their ability to degrade EZH2. We will also carry out medicinal chemistry optimization of the screening hits and astemizole we have identified in our alphascreen HTS. Further characterization of binding will be done using additional biophysical and biochemical techniques. We will also test compounds for their effects on prostate cancer in different cellular systems. Promising inhibitors will also begin to be screened for their pharmaceutical properties such as solubility and metabolic stability.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have shown that compounds that covalently bind to EZH2 cause protein degradation, which suggests unknown mechanisms by which EZH2 is regulated in prostate cancer cells. We have also shown that PROTACS can be applied to degrade EZH2, which would be the first demonstration of this strategy applied to EZH2. Our HTS inhibitors inhibit EZH2/EED binding but do not bind to the alpha-helix site on EED, suggesting other critical sites of interaction between the two proteins that may affect PRC complex stability.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Nothing to report.

Remember that significant changes in objectives and scope require prior approval of the agency.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

We completed Major Task #7: "Complete virtual high-throughput screen (vHTS) to identify novel EZH2/EED inhibitors" ahead of schedule. For this major task, we identified two separate druggable sites on EED that we intended to find molecule to bind. This binding would prevent interaction with EZH2 and lead to the degradation of EZH2. For each of the two vHTS screens, we identified sets of 30-50 compounds that appeared possible to bind. However, after acquiring these compounds and testing in our in vitro assays, none of the compounds prevented EZH2/EED binding as predicted by our in silico model.

Because our vHTS failed to provide inhibitor hits, we used the AlphaScreen assay we had developed to carry out an in vitro HTS. We tested ~ 20,000 drug-like small molecules for their ability to disrupt binding between EED and EZH2. Compounds active in this assays were validated by eliminating assay artifacts (i.e. false positives) and confirming their potency by testing over a dose-range. This resulted in a set of >10 compounds that have been validated to inhibit the binding between EED and EZH2. Work to improve their potency and further characterize their binding is underway.

We also pursued two additional strategies in parallel to develop compounds that cause degradation of EZH2. The first is through the use of PROTACS (see section 3 above for details). In this approach, we are making new compounds that bind EZH2 and recruit an E3 ligase to promote polyubiquitination and subsequent proteasomal degradation. In the second strategy, we are making covalent EZH2 inhibitors. It has been shown that compounds that covalently bind EZH2 can cause degradation. We are making novel analogs that appear to be acting as covalent inhibitors and we have observed significant EZH2 protein degradation by treating cells with them. We are making new analogs of our PROTACS and covalent inhibitors to further improve their biological activity and characterize their mechanism of action.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

The postdoctoral fellow performing chemistry and in vitro screening, Dr. Corinne Ley, did not start until 3 months into the project. During this period, we were focused on Major Task #7, the vHTS. This delayed start reduced our personnel expenses in year 1. Because our vHTS failed to identify EED/EZH2 inhibitors, we carried out an in vitro HTS as an alternative approach, which has succeeded in delivering novel hits. However, this in vitro HTS resulted in additional unforeseen costs. The extra costs for the HTS were roughly similar to the amount we saved on personnel costs by the delayed start of Dr. Ley.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Poster presented by Dr. Corinne Ley at the Northwestern University symposium titled "The Chemical Impact on Life Sciences Colloquium & Scientific Poster Session", held at Northwestern University. Dr. Ley was awarded first place in the postdoctoral fellow category for her poster presentation.

- *List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.*

Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: Gary E. Schiltz
Project Role: Partnering Principal Investigator
Researcher Identifier (ORCID ID): 0000-0003-4180-5051
Nearest person month worked: 3

Contribution to Project: Dr. Schiltz is directing the molecular development of the new EZH2 inhibitors. This includes overseeing the molecular modeling, medicinal chemistry, and several in vitro screening assays being carried out by Drs. Ley and Mishra. He is helping to design experiments and decide on how to achieve our goal of developing new agents targeting EZH2. He is working closely with his partner PI Dr. Yu to analyze biological data.

Funding Support: N/A

Name: Rama K. Mishra, PhD
Project Role: Co-Investigator
Researcher Identifier (ORCID ID): N/A
Nearest person month worked: 2

Contribution to Project: Dr. Mishra is carrying out the molecular modeling for this project. This includes conducting in silico screening to identify new EZH2/EED inhibitors, modeling the binding of new small molecules to EZH2 and/or EED proteins, and analysis of the drug-like properties of new compounds.

Funding Support: N/A

Name: Corrine Ley
Project Role: Postdoctoral Fellow
Researcher Identifier (ORCID ID): N/A
Nearest person month worked: 9

Contribution to Project: Dr. Ley is carrying out the synthetic and medicinal chemistry for the project, as well as conducting the biochemical in vitro screens to test the bioactivity of new compounds. She is synthesizing new analogs of our screening hits, preparing new covalent inhibitors and PROTACS, and testing the new molecules for their ability to inhibit the binding between EZH2 and EED.

Funding Support: N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

New grants started in last reporting period:

Prostate Cancer Foundation (PIs: Drs. Jindan Yu and Maha Hussein)
Targeting chemokine signaling and MAPK/ERK pathway in advanced prostate cancer
The immediate goal is to examine CXCR7-MAPK-ERK activation in mCRPC patient specimens and to test the efficacy of Trametinib and Enz combination in delaying the progression of PCa to CRPC in first-line treatments or the development of Enz resistance in second-line treatments. The long term goal is to develop a front-line trial to test whether trametinib prevents the fatal transition of PCa to mCRPC. Our work will have significant impact on aggressive PCa and mCRPC patients.
Dr. Schiltz is a co-investigator on this grant.

NIH/NIDDK P30DK114857 (PIs: Drs. Quaggin, Scheidt, and George)
Kidney Therapeutics: Translating Discoveries into Prevention, Treatment and Cures for Kidney Diseases
The overall theme of the Northwestern George M. O'Brien Kidney Core Center named NU-GoKIDNEY, will focus on Kidney Therapeutics to translate discoveries into prevention, treatment and cures.
Dr. Schiltz is a co-investigator on this grant.

Stephen M Coffman Charitable Trust (PI: Dr. Alex Stegh)
Identification of small molecule inhibitors for IDH1
The enzyme IDH1 has been shown to be involved in glioblastoma multiforme (GBM). The goal of this project is to identify and develop new small molecules that inhibit wild-type IDH1 enzyme. These compounds will be tested for their ability to inhibit enzyme activity and their effects on GBM.
Dr. Schiltz is a co-investigator on this grant

Grants ended in last reporting period:

NIH/NCI R01CA189074-03 (PIs: Drs. Schiltz, Miller, and Platanias)
Small molecule CXCR4 modulators as molecular probes for studying AML
In this project, we will optimize CXCR4 modulators to create more potent and drug-like probes to study CXCR4-related biology.
Dr. Schiltz was the contact principal investigator on this grant

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have

provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

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